Exhibit 3

11/21/2007

c-di-GMP is an Effective Immunomodulator and Immunostimulatory Molecule Against Pneumococcal Infection

Abiodun D. Ogunniyi,[1] James C. Paton,[1] Alun C. Kirby,[2] Jonathan A. McCullers,[3] Jan Cook,[1] Mamoru Hyodo,[4] Yoshihiro Hayakawa,[4] and David K. R. Karaolis[5, 6*]

[1]School of Molecular and Biomedical Science, University of Adelaide, South Australia 5005, Australia, [2]Department of Biology, University of York, York YO10 5YW United Kingdom, [3]Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN 38104, USA, [4]Graduate School of Information Science, Nagoya University, Nagoya, Japan, [5]Intragenics Research Institute, Havre de Grace, MD 21078, USA and [6]Karagen Pharmaceuticals, Baltimore, MD 21210, USA.

Running title: c-di-GMP is an immunostimulatory molecule

Potential conflicts of interest: D.K.R. Karaolis has three related patents: a method for attenuating virulence of microbial pathogens and for inhibiting microbial biofilm formation (PCT/US04/23498); a method for stimulating the immune, inflammatory, or neuroprotective response (U.S. 11/079, 886; PCT/ US05/08447); and a method for inhibiting cancer cell proliferation or increasing cancer cell apoptosis (U.S. 11/079,779; PCT/US05/08448).

Key words: *Streptococcus pneumoniae*, c-di-GMP, protein vaccines, adjuvant, immunomodulator, invasive pneumococcal disease, pneumolysin, PspA, innate immunity, adaptive immunity

Word counts: Abstract, 201 words.

*Corresponding author. Mailing address: Intragenics Research Institute, 415 Oakington Road, Havre de Grace, MD 21078. Phone: 410-522 8698. Email: dkaraolis@intragenics.org

Summary

Streptococcus pneumoniae **is the commonest cause of vaccine-preventable deaths in children under 5 years of age. Current global efforts are focused on alternative prophylactic strategies against pneumococcal disease. It was previously demonstrated that cyclic diguanylate (c-di-GMP) is a unique bacterial intracellular signaling molecule capable of stimulating protective innate immunity against various bacterial infections. In this work, the effects of intranasal pretreatment with c-di-GMP before challenge, or intraperitoneal coadministration of c-di-GMP with the pneumolysin toxoid (PdB) or PspA before challenge with pneumococci was investigated in mice. Intranasal pretreatment with c-di-GMP resulted in significant decrease in bacterial load in lungs and blood after serotypes 2 and 3 challenge, and significant decrease in lung titers after serotype 4 challenge. Potential cellular mediators of these protective responses were identified in lungs and draining lymph nodes. Intraperitoneal coadministration of c-di-GMP with PdB or PspA before challenge resulted in significantly higher antigen-specific antibody titers and increased survival of mice, compared to that obtained with alum adjuvant. These findings demonstrate that local or systemic c-di-GMP administration stimulates protective innate and adaptive immunity against invasive pneumococcal disease. We propose that c-di-GMP can be used as an effective broad spectrum immunomodulatory molecule and vaccine adjuvant to prevent various infectious diseases.**

INTRODUCTION

Streptococcus pneumoniae is the leading cause of bacterial pneumonia, meningitis, and otitis media in the United States (7). In spite of the availability of antimicrobials, the capsular polysaccharide (PS) vaccine, and the 7-valent protein-PS conjugate vaccine, pneumococcal disease continues to be responsible for high morbidity and mortality worldwide, especially in groups at high risk (36). Consequently, global efforts are focused on exploring alternative pneumococcal vaccine strategies to address the shortcomings of existing formulations, without compromising efficacy. One of these approaches involves the development of vaccines based on pneumococcal proteins that contribute to pathogenesis and are common to all serotypes. To date, the most promising vaccine candidates are the pneumolysin toxoid (PdB), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC, also referred to as choline binding protein A) and the 37-kDa metal-binding lipoprotein PsaA (reviewed by (36)).

We have shown that c-di-GMP (3',5'-cyclic diguanylic acid or cyclic diguanylate or cGpGp) initially identified in the bacterium *Acetobacter xylinum*, is an intracellular signaling molecule (1, 42, 43). It is present in multiple bacterial species but not in eukaryotes (9, 13, 17, 38-40), and is now recognized to control many key functions in bacteria, including survival, adherence, colonization, and biofilm formation (8, 15, 17, 23, 38, 41). Recent studies indicate that it might also modulate host cellular responses (23), resulting in an enhanced control of infection (6). Consistent with this notion, we recently demonstrated that c-di-GMP increases MIG/CXCL9 (a chemoattractant for activated T cells) suggesting possible antitumor activity (21), and inhibits basal and growth factor-induced proliferation of human colon carcinoma cells (20).

Recently, c-di-GMP was shown to modulate the immune system to prevent and fight various lethal bacterial infections (21). Intranasal (i.n.) or subcutaneous administration of c-di-GMP before intratracheal challenge with *Klebsiella pneumoniae* also resulted in significantly increased survival and reduction in bacterial counts in lung and blood (22). The response was characterized by enhanced accumulation of neutrophils, αβ T cells, and activated NK and αβ T lymphocytes, associated with earlier and more vigorous expression of chemokines and type-1 cytokines (22). Moreover, lung macrophages recovered from *Klebsiella*-infected mice pretreated with c-di-GMP expressed greater quantities of iNOS (inducible nitric oxide synthase) and nitric oxide *ex vivo* than those isolated from mice pretreated with control cGMP (22). These findings demonstrate that c-di-GMP delivered locally or systemically stimulates protective innate immunity in the lung and protects mice against bacterial invasion.

In this study, we investigated the ability of c-di-GMP to protect against systemic pneumococcal infection, using established mouse models. We provide additional direct evidence that c-di-GMP is immunostimulatory, can protect against infection, and acts as an effective vaccine adjuvant against systemic disease.

MATERIALS AND METHODS

Bacterial strains. The pneumococcal strains used in this study were D39, a virulent type 2 strain (2), strain T4, a type 4 encapsulated strain (45), and Xen10, a bioluminescent derivative of type 3 strain A66.1 (12) that has been engineered to express luciferase so infections can be followed using bioluminescent imaging (Xenogen Corp.,Hopkinton, MA).

Mice. Intranasal challenge studies with D39 and T4 were carried out using 6-8-week old female Balb/cByJ mice (Jackson Laboratory, Bar Harbor, ME) at St. Jude Children's Research Hospital. For bioluminescence studies, 6-8-week old female C57BL/6 mice (Charles River, Margate, U.K.), were used at the University of York. For i.p. active immunization/challenge studies, 5-week old male outbred CD1 (Swiss) mice were used at the University of Adelaide. All animal experiments were approved by the Animal Care and Use and Ethics committees of the various institutions.

c-di-GMP. The c-di-GMP used in these studies was chemically synthesized and prepared as described previously (16). Control cGMP was purchased from Sigma (St. Louis, MO). c-di-GMP and control cGMP were reconstituted at the appropriate concentration in sterile 0.9% NaCl (saline). Control groups received either saline alone or control cGMP (Sigma). All c-di-GMP preparations were free of endotoxin contamination (21), and did not have any direct antimicrobial effects on *S. pneumoniae* (not shown).

Preparation of antigens. A 43 kDa N-terminal His_6-tagged PspA fragment was cloned, expressed, and purified as described previously (4, 32, 33). Pneumolysin toxoid (PdB) was cloned as a His_6-tagged fusion protein from plasmid pJCP202 (27) by amplification with primers AD20 5'-GGAACTTATTAGGATCCAGAAGATGGC-3' and AD21 5'-TTGTCGCGAGCTCTCTCCTCTCCTA-3' (*Bam*HI and *Sac*I restriction sites underlined). The

PCR fragment was cloned into the corresponding restriction sites in the polylinker of pQE32 (Qiagen Inc.) and then used to transform *Escherichia coli* BL21 (DE3). Recombinant His_6-PdB was expressed, purified and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining, as described previously (34). The protein migrated at approx. 53 kDa, consistent with its predicted size from the DNA sequence. His_6-PspA fragment was analyzed in parallel and also migrated at the expected size (approx. 43 kDa). Both antigens were judged to be >95% pure (not shown).

Intranasal challenge. For bioluminescence studies, mice were challenged i.n. with 2×10^6 CFU of Xen10 in a 50 µl volume under light anesthesia with 2.5% isoflurane (Baxter Healthcare Corporation, Deerfield, IL). Before challenge, groups of mice were treated i.n. with either c-di-GMP (200 nmol), cGMP (200 nmol; Sigma) or PBS at -48 h and -24 h. Infection was then followed in individual mice using the IVIS Imaging 100 system (Xenogen). Luminescence (photons/sec/cm^2) was determined at various time points and quantified using LivingImage software (Xenogen).

For studies using c-di-GMP pretreatment to protect against i.n. challenge and to enumerate bacterial titers in the lungs and blood, mice were anesthetized 48 and 24 h before infection and given 200 nmol per mouse of either c-di-GMP, or control cGMP (Sigma). Both were diluted in saline and delivered in a final volume of 50 µl (25 µl per nostril). Before infection, mice were anesthetized and then challenged with 1×10^4, 1×10^5, or 1×10^6 CFU of D39 or 1×10^5 CFU of T4 in 100 µl sterile PBS (50 µl per nostril). Twenty-four hours after infection, mice were lightly anesthetized with isoflurane and approx. 100 µl of blood was obtained via retro-orbital puncture. Mice were then euthanized by CO_2 inhalation followed by cervical dislocation. Lungs were removed aseptically, washed three times in sterile PBS, and

homogenized in 500 µl sterile PBS. Pneumococci were enumerated by plating serial 10-fold dilutions of lung homogenates or blood on tryptic soy agar (Difco) plates supplemented with 3% v/v sheep erythrocytes.

Immunization of mice. Six groups of 5-to 6-week-old male CD1 mice (15 per group) were immunized intraperitoneally (i.p.) with PdB or PspA, in either alum adjuvant (Imject Alum no. 77161; Pierce, Rockford, Ill.) or c-di-GMP. Each group received either PdB+alum, PspA+alum, PdB+ c-di-GMP, PspA+c-di-GMP, or a placebo. Each mouse received three doses of 10 µg of each antigen in either 100 µg of alum or 200 nmol of c-di-GMP at 14-day intervals. Mice given the placebo received an identical course of saline plus alum or c-di-GMP. Sera were collected from individual mice by retro-orbital bleeding 1 week after the third immunization.

ELISA and Western blotting. Aliquots of sera from individual mice in each group were pooled and assayed for protein-specific antibodies by enzyme-linked immunosorbent assay (ELISA), using 96-well polystyrene microtiter trays (Nunc) coated with purified antigens. Pooled sera were analyzed for total IgG and IgG subclass (IgG1, IgG2a) antibodies, while sera from individual mice were assayed for total IgG, as described previously (33, 34). Pooled sera were also subjected to Western immunoblotting using whole-cell lysates of D39 or purified proteins as the antigen, as described previously (34).

Intraperitoneal challenge. Mice were challenged 2 weeks after the third immunization using opaque-phase variant of D39, selected on THY-catalase plates (48). Bacteria were grown as described previously (33) and the dose adjusted to 3×10^2 CFU (approx. 30 LD_{50} dose of D39 for CD1 mice). Mice were closely monitored for 21 days, and survival times recorded.

Flow cytometry. Preparation of single cell suspensions of lung and draining lymph nodes (dLN), and subsequent flow cytometric staining were carried out as previously described

(26, 27). Total viable cell counts were determined by trypan blue exclusion. The following antibodies were used (all BD Pharmingen, Oxford, UK, unless otherwise stated): HL3 (CD11c), RB6-8C5 (Ly6G/Ly6C; Gr1), 2G9 (MHC-II), M1/70 (CD11b), 7/4 (7/4 antigen; Serotec, Oxford, U.K.), 145.2C11 (CD3), H57-597 (TCR b), GK1.5 (CD4), 53-6.7 (CD8a), RA3.6B2 (B220), 1D3 (CD19), 3/23 (CD40) and GL1 (CD86). All samples were treated with anti-FcR (2.4G2) prior to specific staining. Samples were acquired on a Cyan ADP flow cytometer.

Statistics. Lung and blood titers were compared by Tukey's analysis of variance, using SigmaStat for Windows (SysStat Software, Inc., V3.11). Differences in luminescent bacterial titers were determined by Student's *t* test. Median survival times of groups in the i.p challenge experiment were compared by the Mann-Whitney *U* test (two-tailed). Antibody titers from individual mice were compared by Student's *t* test (two-tailed). Data from flow cytometry were analyzed with Summit 4.3 software (Dako, Ely, U.K.). A P value of <0.05 was considered statistically significant.

RESULTS

c-di-GMP enhances innate protection against lethal pneumococcal infection. Previous Xenogen mouse model of pneumococcal infection indicated that i.n. infection with 1-2 $\times 10^6$ CFU of *S. pneumoniae* Xen10 typically establishes a lung-restricted lethal infection (12, 35) while allowing disease progression to be followed from as early as 5 min post challenge (26). To examine the effects of c-di-GMP on host survival and bacterial growth in this model, mice were pretreated at 48 h and 24 h with c-di-GMP, cGMP or PBS (10 mice per group) before i.n. challenge with a lethal dose of Xen10 and imaged at various time points thereafter.

At early time points (1-4 h post-challenge), mice pretreated with PBS or cGMP showed a general tendency towards control of bacterial numbers, reflected by relatively small increases in luminescence (Fig. 1A, B). However, c-di-GMP-pretreated mice generally showed rapidly increasing luminescence, suggesting an inability to control bacterial growth at this stage. Surprisingly, from 8 h post-infection, c-di-GMP-pretreated mice exerted a rapid and effective control over bacterial growth. Instead of continued multiplication, luminescence in lungs of the mice tended to plateau, followed by a much slower increase in bacterial titers over the following 24-48 h (Fig. 1B, C). Mice in both control groups, despite their lower bacterial loads at early time points, appeared to succumb to infection before 18 h post-challenge. In these groups, luminescence increased significantly in the lungs (Fig. 1B, C-F) compared to c-di-GMP-treated mice at 42 and 48 h. Together, these data suggest that, despite a short initial lag, c-di-GMP pretreatment is beneficial to the host and aids in controlling fulminant pneumococcal infection.

Pneumococcal counts in lung and blood of pretreated mice. To test the effect of c-di-GMP on invasive disease by virulent strains, groups of 6 mice were infected with 1×10^6 CFU of *S. pneumoniae* D39 or 1×10^5 CFU of T4. At 24 h post-infection, bacteria were enumerated from

blood cultures and lung homogenates. Mice treated twice with c-di-GMP 48 and 24 h before D39 challenge had greater than 100-fold reduction in mean lung titers compared to mice treated with cGMP (Fig. 2A). Mice challenged with T4 also showed significant reductions in mean lung titers. Blood titers were also lower in both groups, but the difference was only statistically significant for D39-challenged mice (Fig. 2B).

To determine whether this effect could be seen across a broader range of infectious doses, groups of mice were treated as above and infected with 1×10^4 or 1×10^5 CFU of D39 (Fig. 2C). Mice treated with c-di-GMP had approx. 100-fold reductions in lung titers at both additional doses tested, although this was only statistically significant in the group challenged with the lower dose.

Analysis of sera. ELISA analysis of pooled sera from groups of mice immunized with PdB and PspA indicated strong, antigen-specific antibody responses, and IgG1 response was predominant (Table 1). Interestingly, antibody titers elicited to PdB+c-di-GMP and PspA+c-di-GMP were significantly higher than PdB+alum and PspA+alum (P=0.003 and P=0.0005, respectively). When antibody titers from 3 mice that survived the challenge and from 3 mice that died soon after challenge in each group were compared, the titers for surviving mice immunized with PdB+alum and PspA+c-di-GMP were significantly higher (P=0.01 in both cases). However, antibody titers for surviving mice immunized with PspA+alum and PdB+c-di-GMP were not significantly different from those obtained from their counterparts that died early.

Western immunoblot analysis of whole cell lysates of D39 also demonstrated protein-specific antibody responses (Fig. 3). Anti-PdB sera specifically labeled pneumolysin, but a characteristic labeled smear was observed with anti-PspA sera (31, 34). Western blot analysis of the purified proteins also demonstrated specific antibody responses (not shown).

Protection studies. In the i.p. challenge experiment, mice immunized with PdB+alum and PspA+alum survived significantly longer than the placebo group [$P<0.02$ in both cases] (Fig. 4). Furthermore, PdB+c-di-GMP-immunized mice survived significantly longer than those immunized with c-di-GMP alone ($P<0.05$). Surprisingly, the median survival time for mice immunized with PspA+c-di-GMP, although longer than for those immunized with c-di-GMP alone (>504 h vs 60 h), did not reach statistical significance. Interestingly, mice immunized with PdB+c-di-GMP survived significantly longer than those that received PdB+alum ($P<0.05$).

c-di-GMP treatment affects both lung and draining LN populations. To examine potential mechanisms underlying the protective effect of c-di-GMP, immune cell populations from lungs and lung draining lymph nodes (dLN) were quantified in naïve, cGMP and c-di-GMP treated mice. Compared with naïve lungs, no significant effect of cGMP ($P=0.17$) or c-di-GMP ($P=0.19$) on overall cell number was observed. Treatment with c-di-GMP reduced both T and B lymphocyte counts in lungs compared with control groups (Fig. 5A), although the proportion of $CD44^{low}$ naïve T cells was slightly increased in c-di-GMP-treated mice compared with naïve ($P=0.01$) and cGMP groups ($P=0.001$). c-di-GMP treatment had no significant effect on total $CD11c^{hi}MHCII^{low}$ alveolar macrophage (AM) numbers (Fig. 5B), but increased the proportion of these cells expressing CD11b (Fig. 5B), suggesting an increase in newly influxed AM (27). Similarly, while numbers of lung $CD11c^{hi}MHCII^{hi}$ dendritic cells (DC) were not significantly influenced by c-di-GMP treatment (data not shown), DC from these mice expressed higher levels of CD40 and CD86 than did DC from either control group (Fig. 5C, D). Finally, neither monocyte nor neutrophil populations in the lungs were significantly altered by c-di-GMP treatment compared with control groups.

In contrast to the lungs, c-di-GMP treatment significantly increased the total cell number of lung dLN compared to cGMP (P=0.01) or naïve groups (P=0.01), reflected in significant increases in T lymphocyte, but not B lymphocyte, numbers in the dLN (Fig. 6A). Furthermore, there was an increase in the proportion of $CD44^{low}$ naïve cells c-di-GMP-treated mice (Fig. 6B). DC population significantly increased in both cGMP and c-di-GMP treated groups compared with controls (Fig. 6C). However, expression of CD86 was significantly increased only in the c-di-GMP group (Fig. 6D), but not CD40 (not shown). Finally, while c-di-GMP treatment had no significant effect on dLN neutrophil counts (Fig. 6E), mean counts of monocytes within the lung dLN of the c-di-GMP group were >20-fold higher than in either control group (Fig. 6E). Taken together, these data demonstrate cellular consequences of c-di-GMP treatment which may facilitate more potent immune responses.

DISCUSSION

In this study, we report that pretreatment of mice either i.n. or i.p. with c-di-GMP, but not control nucleotide (cGMP) or saline, can induce a significant protective immune response against i.n. or i.p. challenge with virulent pneumococci in various murine infection models. Our current findings are consistent with previous studies with *Staphylococcus aureus* and *K. pneumoniae*, as well as recent *in vivo* and *in vitro* studies showing that c-di-GMP is immunomodulatory and immunostimulatory (6, 21, 22).

In bacterial pneumonia, clearance of pathogens is primarily dependent upon a vigorous innate immune response (5, 28, 46, 47). The present study clearly demonstrates that c-di-GMP administration enhances several key aspects of the cytokine-mediated innate immunity in the lung. Although the cellular components of c-di-GMP-stimulated immunity have not been clearly defined, several candidate cell populations, such as neutrophils, MIP-2, NK, NKT and αβ T cells, are likely to be involved, as shown previously (22). Neutrophils are important for bacterial clearance from the lung (28, 47), and c-di-GMP-induced upregulation of MIP-2 may contribute to increased neutrophil trafficking. Moreover, NK cells are considered to be the primary source of IFN-γ in the lung early during bacterial infection (10, 11), while NKT cells can be primed to secrete prodigious quantities of IFN-γ during infection (14, 24, 44), as well as contributing to innate immunity against pulmonary pneumococcal challenge (25). The promotion of enhanced type 1 immunity in response to c-di-GMP administration supports the possibility that c-di-GMP directly stimulates DC mediated responses in the lung via activation of p38 MAPK (mitogen activated protein kinase) and enhanced IL-12 p40 expression, as demonstrated recently (21). However, other cells, including lung macrophages, may also contribute.

We also demonstrate that c-di-GMP can prime or directly stimulate multiple beneficial aspects of innate and adaptive immunity. Therefore, its use avoids current concerns with drug resistance associated with traditional antibiotics. Since c-di-GMP has fast-acting broad spectrum activity and flexibility in route of administration, its use may also be complementary to antibiotics (prophylactically or therapeutically), as previously proposed (21).

In the bioluminescence studies, c-di-GMP pretreatment resulted in reduced early control of bacterial numbers at 0-4 h, but a significant reduction in bacterial titers at 42-48 h. Although the reasons for these observations are unclear, mice pretreated with c-di-GMP, but not with cGMP or saline, rapidly exhibited physical symptoms of mild toxic shock (piloerection, severe sweating) following challenge (unpublished observations). This suggests a rapid pro-inflammatory cytokine response initiated by pneumococcal challenge in c-di-GMP 'primed' mice which did not occur in control groups. While it was previously shown that TNF, a major pro-inflammatory cytokine involved in toxic shock responses, is neither significantly induced nor required for a complete cellular response to *S. pneumoniae* (26), it remains to be seen whether such a locally initiated shock response may be detrimental to early cellular responses in the lungs. It was striking that after approximately 8 h, c-di-GMP-pretreated mice exerted control over bacterial numbers. Since early anti-pneumococcal responses in previously naïve mice are dominated by neutrophils and other phagocytes (26), we propose that c-di-GMP pretreatment results in the ability to mount a more effective and prolonged phagocytic response. This may occur either through increased phagocyte recruitment or more efficient phagocytic killing (22). These possibilities and the mechanism involved are under investigation.

Although the data using the serotype 3 strain Xen10 were compelling, different pneumococcal strains often behave quite differently in mice (35). We therefore sought to

determine whether amelioration of invasive disease was possible in strains with pathogenicity profiles distinct from Xen10. While Xen10 stays confined to the lung, the type 2 strain D39 causes both lung infection and a high grade sepsis with bacteremia, while the type 4 strain T4 causes primary bacteremia which progresses to meningitis in untreated mice; with seeding of the lungs occurring secondary to bacteremia (35). With both these virulent strains, pretreatment with c-di-GMP decreased lung and blood titers compared to mock treated controls, indicating the effects are not specific to the lung and can be generalized to several pneumococcal strains and serotypes. This provides additional support for the proposed mechanism suggesting these results with *S. pneumoniae* (and our previous results with *S. aureus* and *K. pneumoniae*) are based on changes to the host's innate immunity, rather than a direct effect on any particular bacterial strain.

In the i.p. immunization/challenge studies, the protection elicited by PdB and PspA is in agreement with previous findings (33, 34, 37). Passive-immunization-i.p.-challenge experiments demonstrated that the protection elicited by PdB and PspA was, at least, partly antibody mediated (33). In this work, mice immunized with PdB and PspA in c-di-GMP elicited significantly higher antibody titers than mice that received the antigens in alum. Indeed, mice immunized with PdB+c-di-GMP were significantly better protected than those immunized with PdB+alum. While high antibody titers correlated with protection, this may not necessarily represent the true or only mechanism of protection. Recent studies in mice have indicated T-cell mediated, antibody-independent immunity against pneumococcal colonization (29, 30) or systemic disease (18, 19). Indeed, low CD4 T-cell immunity to pneumolysin was found to be associated with pneumococcal carriage in children (49), while PspA was effective at eliciting T cell-mediated responses during invasive disease in adults (3). Therefore, protection afforded by

either PdB+c-di-GMP or PspA+c-di-GMP may be T-cell mediated, although this remains to be demonstrated directly.

In conclusion, we demonstrate that innate immune stimulation with c-di-GMP ameliorates subsequent invasive pneumococcal disease and augments the immune response. Furthermore, the fact that the results are more generalizable and reproducible in different serotypes, and complementary in different infection models, is very encouraging. These findings, in addition to the possibility that formulation of vaccines with c-di-GMP is likely to be simpler, argues in favor of using c-di-GMP as an adjuvant of choice. This would make deployment of such vaccines to developing countries, where the need is greatest, more affordable. We propose that c-di-GMP treatment may play a potentially beneficial role in immunoprophylaxis by activating innate host defenses against respiratory and systemic infections. Potential clinical applications for c-di-GMP include its use alone or in combination with other pharmaceuticals as an immunostimulatory, immunoprophylactic, and immunotherapeutic agent or vaccine adjuvant to prevent a variety of infections.

Acknowledgments

The authors wish to acknowledge excellent technical support from Amy R. Iverson at St. Jude Children's Research Hospital. D.K.R.K. is a recipient of a Burroughs Wellcome career Award in the Biomedical Sciences. This research was supported by the Army Research Office Grant to D.K.R.K. and National Health and Medical Research Council of Australia Program Grant 284214.

REFERENCES

1. **Amikam, D., and M. Benziman.** 1989. Cyclic diguanylic acid and cellulose synthesis in *Agrobacterium tumefaciens*. J. Bacteriol. **171**:6649-6655.

2. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. **79**:137-158.

3. **Baril, L., J. Dietemann, M. Essevaz-Roulet, L. Béniguel, P. Coan, D. E. Briles, B. Guy, and G Cozon.** 2006. Pneumococcal surface protein A (PspA) is effective at eliciting T cell-mediated responses during invasive pneumococcal disease in adults. Clin. Exp. Immunol. **145**:277-286.

4. **Briles, D. E., S. K. Hollingshead, J. King, A. Swift, P. A. Braun, M. K. Park, L. M. Ferguson, M. H. Nahm, and G. S. Nabors.** 2000. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J. Infect. Dis. **182**:1694-1701.

5. **Broug-Holub, E., G. B. Toews, J. F. van Iwaarden, R. M. Strieter, S. L. Kunkel, R. Paine 3rd, and T. J. Standiford.** 1997. Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella* pneumonia: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. Infect. Immun. **65**:1139-1146.

6. **Brouillette, E., M. Hyodo, Y. Hayakawa, D. K. R. Karaolis, and F. Malouin.** 2005. 3',5'-cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus*

strains in a mouse model of mastitis infection. Antimicrob. Agents Chemother. **49:**3109-3113.

7. **CDC.** 2002. Assessment of Susceptibility Testing Practices for *Streptococcus pneumoniae* -United States, February 2000. MMWR **51:**393-394.

8. **D'Argenio, D. A., M. W. Calfee, P. B. Rainey, and E. C. Pesci.** 2002. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. J. Bacteriol. **184:**6481-6489.

9. **D'Argenio, D. A., and S. I. Miller.** 2004. Cyclic di-GMP as a bacterial second messenger. Microbiology. **150:**2497-2502.

10. **Deng, J. C., K. Tateda, X. Zeng, and T. J. Standiford.** 2001. Transient transgenic expression of gamma interferon promotes *Legionella pneumophila* clearance in immunocompetent hosts. Infect. Immun. **69:**6382-6390.

11. **Ferlazzo, G., B. Morandi, A. D'Agostino, R. Meazza, G. Melioli, A. Moretta, and L. Moretta.** 2003. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. Eur. J. of Immunol. **33:**306-313.

12. **Francis, K. P., J. Yu, C. Bellinger-Kawahara, D. Joh, M. J. Hawkinson, G. Xiao, T. F. Purchio, M. G. Caparon, M. Lipsitch, and P. R. Contag.** 2001. Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumoniae* transformed with a novel gram-positive *lux* transposon. Infect. Immun. **69:**3350-3358.

13. **Galperin, M. Y., A. N. Nikolskaya, and E. V. Koonin.** 2001. Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol. Lett. **203:**11-21.

14. **Gonzalez-Aseguinolaza, G., C. de Oliveira, M. Tomaska, S. Hong, O. Bruna-Romero, T. Nakayama, M. Taniguchi, A. Bendelac, L. Van Kaer, Y. Koezuka, and M. Tsuji M.** 2000. α-Galactosylceramide-activated Vα14 natural killer T cells mediate protection against murine malaria. Proc. Natl. Acad. Sci. USA. **97:**8461-8466.

15. **Hecht, G. B., and A. Newton.** 1995. Identification of a novel response regulator required for the swarmer-to-stalked-cell transition in *Caulobacter crescentus*. J. Bacteriol. **177:**6223-6229.

16. **Hyodo, M., Y. Hayakawa, and D. K. R. Karaolis.** 2006. Organic synthesis, chemical properties, and biological activities of cyclic bis(3'-5') diguanylic acid (c-di-GMP) and its analogs. J. Synth. Org. Chem. Jpn. **64:**359-370.

17. **Jones, H. A., J. W. Lillard Jr., and R. D. Perry.** 1999. HmsT, a protein essential for expression of the haemin storage (Hms+) phenotype of *Yersinia pestis*. Microbiology. **145:**2117-2128.

18. **Kadioglu, A., W. Coward, M. J. Colston, C. R. Hewitt, and P. W. Andrew.** 2004. CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. Infect. Immun. **72:**2689-2697.

19. **Kadioglu, A., N. A. Gingles, K. Grattan, A. Kerr, T. J. Mitchell, and P. W. Andrew.** 2000. Host cellular immune response to pneumococcal lung infection in mice. Infect. Immun. **68:** 492–501.

20. **Karaolis, D. K. R., K. Cheng, M. Lipsky, A. Elnabawi, J. Catalano, M. Hyodo, Y. Hayakawa, and J.-P. Raufman.** 2005. 3',5'-Cyclic diguanylic acid (c-di-GMP) inhibits basal and growth factor-stimulated human colon cancer cell proliferation. Biochem. Biophys. Res. Commun. **329:**40-45.

21. **Karaolis, D. K. R., T. K. Means, D. Yang, M. Takahashi, T. Yoshimura, E. Muraille, D. Philpott, J. T. Schroeder, M. Hyodo, Y. Hayakawa, B. G. Talbot, E. Brouillette, and F. Malouin.** 2007. Bacterial c-di-GMP is an immunostimulatory molecule. J. Immunol. **178:**2171-2181.

22. **Karaolis, D. K. R., M. W. Newstead, X. Zeng, M. Hyodo, Y. Hayakawa, U. Bhan, H. Liang, and T. J. Standiford.** 2007. c-di-GMP stimulates protective innate immunity in bacterial pneumonia. Infect. Immun. **75:**4942-4950.

23. **Karaolis, D. K. R., M. H. Rashid, C. Rajanna, W. Luo, M. Hyodo, and Y. Hayakawa.** 2005. c-di-GMP (3'-5'-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. Antimicrob. Agents Chemother. **49:**1029-1038.

24. **Kawakami, K., Y. Kinjo, S. Yara, Y. Koguchi, K. Uezu, T. Nakayama, M. Taniguchi, and A. Saito.** 2001. Activation of $V\alpha14^+$ natural killer T cells by α-galactosylceramide results in development of Th1 response and local host resistance in mice infected with *Cryptococcus neoformans*. Infect. Immun. **69:**213-220.

25. **Kawakami, K., N. Yamamoto, Y. Kinjo, K. Miyagi, C. Nakasone, K. Uezu, T. Kinjo, T. Nakayama, M. Taniguchi, and A. Saito.** 2003. Critical role of $V\alpha14^+$ natural killer T cells in the innate phase of host protection against *Streptococcus pneumoniae* infection. Eur. J. Immunol. **33:**3322-3330.

26. **Kirby, A. C., J. G. Raynes, and P. M. Kaye.** 2005. The role played by tumor necrosis factor during localized and systemic infection with *Streptococcus pneumoniae*. J. Infect. Dis. **191:**1538-1547.

27. **Kirby, A. C., J. G. Raynes, and P. M. Kaye.** 2006. CD11b regulates recruitment of alveolar macrophages but not pulmonary dendritic cells after pneumococcal challenge. J. Infect. Dis. **193:**205-213.

28. **Lipscomb, M. F., J. M. Onofrio, and E. J. Nash.** 1983. A morphological study of the role of phagocytes in the clearance of *Staphylococcus aureus* from the lung. J. Reticuloend. Soc. **33:**429-442.

29. **Malley, R., K. Trzcinski, A. Srivastava, C. M. Thompson, P. W. Anderson, and M. Lipsitch.** 2005. $CD4^+$ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proc. Natl. Acad. Sci. USA. **102:**4848-4853.

30. **McCool, T. L., and J. N. Weiser.** 2004. Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. Infect. Immun. **72:**5807-5813.

31. **McDaniel, L. S., J. S. Sheffield, P. Delucchi, and D. E. Briles.** 1991. PspA, a surface protein of *Streptococcus pneumoniae*, is capable of eliciting protection against pneumococci of more than one capsular serotype. Infect. Immun. **59:**222-228.

32. **Nabors, G. S., P. A. Braun, D. J. Herrmann, M. L. Heise, D. J. Pyle, S. Gravenstein, M. Schilling, L. M. Ferguson, S. K. Hollingshead, D. E. Briles, and R. S. Becker.** 2000. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies. Vaccine **18:**1743–1754.

33. **Ogunniyi, A. D., R. L. Folland, D. E. Briles, S. K. Hollingshead, and J. C. Paton.** 2000.

Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. Infect. Immun. **68:**3028-3033.

34. **Ogunniyi, A. D., M. Grabowicz, D. E. Briles, J. Cook, and J. C. Paton.** 2007. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. Infect. Immun. **75:** 350-357.

35. **Orihuela, C. J., G. Gao, M. McGee, J. Yu, K. P. Francis, and E. I. Tuomanen** 2003. Organ-specific models of *Streptococcus pneumoniae* disease. Scand. J. Infect. Dis. **35:** 647-652.

36. **Paton, J. C.** 2004. New Pneumococcal Vaccines: Basic Science Developments, p. 382–402. *In* E. I. Tuomanen, T. J. Mitchell, D. A. Morrison, and B. G. Spratt (ed.), The Pneumococcus. ASM Press, Washington, D.C.

37. **Paton, J. C., R. A. Lock, C.-J. Lee, J. P. Li, A. M. Berry, T. J. Mitchell, P. W. Andrew, D. Hansman, and G. J. Boulnois.** 1991. Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to *Streptococcus pneumoniae* type 19F polysaccharide. Infect. Immun. **59:**2297-2304.

38. **Rashid, M. H., C. Rajanna, A. Ali, and D. K. R. Karaolis.** 2003. Identification of genes involved in the switch between the smooth and rugose phenotypes of *Vibrio cholerae*. FEMS Microbiol. Lett. **227:**113-119.

39. **Römling, U., and D. Amikam.** 2006. Cyclic di-GMP as a second messenger. Curr. Opin. Microbiol. **2:**218-228.

40. **Römling, U., M. Gomelsky, and M. Y. Galperin.** 2005. C-di-GMP: the dawning of a novel bacterial signalling system. Mol. Microbiol. **57:**629-639.

41. **Römling, U., M. Rohde, A. Olsen, S. Normark, and J. Reinkoster.** 2000. AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella typhimurium* regulates at least two independent pathways. Mol. Microbiol. **36:**10-23.

42. **Ross, P., R. Mayer, H. Weinhouse, D. Amikam, Y. Huggirat, M. Benziman, E. de Vroom, A. Fidder, P. de Paus, L. A. Sliedregt, et al.** 1990. The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives. J. Biol. Chem. **265:**18933-18943.

43. **Ross, P., H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinberger-Ohana, R. Mayer, S. Braun, E. de Vroom, G. A. van der Marel, J. H. van Boom, and M. Benziman.** 1987. Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. Nature **325:**279-281.

44. **Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao.** 2003. The regulatory role of Vα14 NKT cells in innate and acquired immune response. Annu. Rev. Immunol. **21:**483-513.

45. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science **293:**498-506.

46. **Toews, G. B., G. N. Gross, and A. K. Pierce.** 1980. The relationship of inoculum size to lung bacterial clearance and phagocytic cell response in mice. Am. Rev. Respir. Dis. **120:**559-566.

47. **Tsai, W. C., R. M. Strieter, B. Mehrad, M. W. Newstead, X. Zeng, and T. J. Standiford.** 2000. CXC chemokine receptor CXCR2 is essential for protective innate host response in murine *Pseudomonas aeruginosa* pneumonia. Infect. Immun. **68:**289-296.

48. **Weiser, J. N., R. Austrian, P. K. Sreenivasan, and H. R. Masure.** 1994. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect. Immun. **62:** 2582-2589.

49. **Zhang, Q., L. Bagrade, J. Bernatoniene, E. Clarke, J. C. Paton, T. J. Mitchell, D. A. Nunez, and A. Finn.** 2007. Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children. J. Infect. Dis. **195:**1194-202.

Figure legends

FIG. 1. The effect of c-di-GMP pretreatment on *S. pneumoniae* Xen10 infection. Luminescence was quantified in the thorax of individual mice given c-di-GMP, cGMP or PBS before *S. pneumoniae* Xen10 infection at: (A) 1, 2.5 and 4 h, (B) 18, 24, 40 and 48 h and (C)1, 4, 18, 24, 40 and 48 h post challenge. Mean (±SEM) luminescences were calculated for each group at each time point. *P<0.01 vs. PBS control group by Student's t-test. (D-F) Luminescence images taken at 48 h post Xen10 challenge. Two mice within the cGMP group died as a result of infection at 24-42 h. In all cases n=5 mice per group. Results are representative of 2 separate experiments.

FIG. 2. Pneumococcal counts in lung and blood of c-di-GMP and cGMP pretreated mice. Groups of mice (n=6) were pretreated with either c-di-GMP or control GMP then infected with 1 × 10^6 CFU of *S. pneumoniae* D39 or 1× 10^5 CFU of T4 intranasally and (A) lung and (B) blood cultures were assayed 24 h later. (C) In a second experiment lung titer data were collected following challenge with either 10^4 or 10^5 CFU of D39. The 25^{th} – 75^{th} percentiles are represented by the shaded box-plots with the horizontal bar indicating the mean value. Error bars indicate the standard deviation of the measurements. An asterisk (*) indicates a significant difference by ANOVA compared to the group treated with c-di-GMP (P<0.05).

FIG. 3. Western blot of whole-cell lysates of *S. pneumoniae* D39 with anti-PdB and anti-PspA sera. The lysates were reacted with specific antisera generated from mice immunized with the indicated antigens. Results for nitrocellulose membranes reacted with sera from mice immunized with alum (lane 1), PdB+alum (lane 2), PspA+alum (lane 3), c-di-GMP (lane 4), PdB+c-di-GMP (lane 5), and PspA+c-di-GMP (lane 6) are shown. The corresponding molecular mass of each protein is indicated by an arrow: pneumolysin (53 kDa), and PspA (86 kDa).

FIG. 4. Survival times for mice after intraperitoneal challenge. Groups of 15 CD1 mice were immunized with the indicated antigens and challenged 2 weeks after the third immunization with approximately 3×10^2 CFU of the capsular type 2 strain D39. The broken lines denote the median survival time for each group.

FIG. 5. Effect of c-di-GMP treatment on lung cells. Lung cell populations were quantified (A, B) in naïve mice (open bars), or following cGMP (closed bars) or c-di-GMP treatment (hatched bars). Graphs show mean numbers of $CD3^+TCRb^+CD19^-$ T cells, $CD3^-TCRb^-CD19^+$ B cells (A), and $CD11c^+MHCII^{low}CD11b^{+/-}$ AM (B). *P<0.05 compared with c-di-GMP group (Student's t-test). Expression of CD40 (C) and CD86 (D) on $CD11c^+MHCII^{high}$ lung dendritic cells is shown for individual mice in each group as mean fluorescence intensity (MFI). Bars represent mean values and numbers indicate P value compared with c-di-GMP group.

FIG. 6. Effect of c-di-GMP treatment on lung draining LN cells. Draining LN populations were quantified (A, C, E) in naïve mice (open bars), or following cGMP (closed bars) or c-di-GMP treatment (hatched bars). Graphs show mean numbers of T and B lymphocyte (A, as defined in Fig. 5), $CD11c^+$ DC (C) and non-overlapping $Gr1^+CD11b^+7/4^+$ neutrophil and monocyte populations, conclusively differentiated based on size and granularity characteristics (E). *P<0.05 compared with c-di-GMP group (A) or naïve group (C) (Student's t test). The percentage of $CD44^{low}$ T cells was in individual mice is shown (B), as is the MFI level of CD86 expression on DCs (D). Bars represent mean values and numbers indicate p value compared with c-di-GMP group.

Table 1. Antibody titers obtained from immunized mice.

Immunogen	ELISA titers[a]					
	Alum-immunized group			c-di-GMP-immunized group		
	Total IgG	IgG1	IgG2a	Total IgG	IgG1	IgG2a
PdB	30,000 (28,200 ± 3,250)	40,000	8,000	60,000 (54,200 ± 5,800)	40,000	25,000
PspA	30,000 (24,700 ± 1,600)	35,000	8,000	140,000 (112,000 ± 17,200)	160,000	50,000
Alum	<500	ND	ND	ND	ND	ND
c-di-GMP	ND	ND	ND	<500	ND	ND

[a]ELISA titers were determined as the reciprocal of the dilution of sera giving 50% of the highest absorbance reading above the background at 405nm. Values in parenthesis represent mean antibody titers ± SEM from six individual mice.

ND= Not Determined.

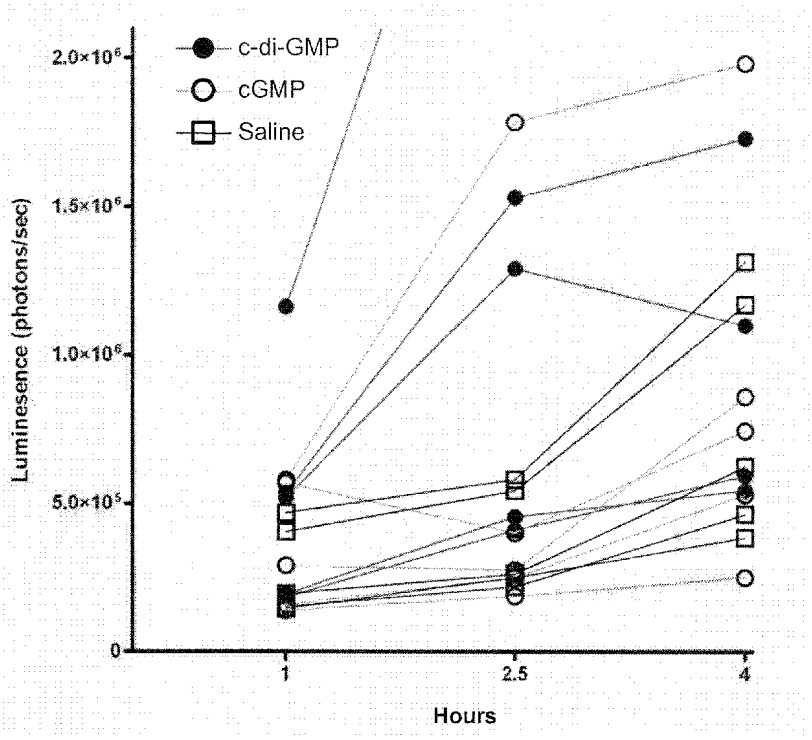

c-di-GMP
cGMP
Saline
2.0×10⁶
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
Luminescence (photons/sec)
1
2.5
4
Hours

Figure 1A

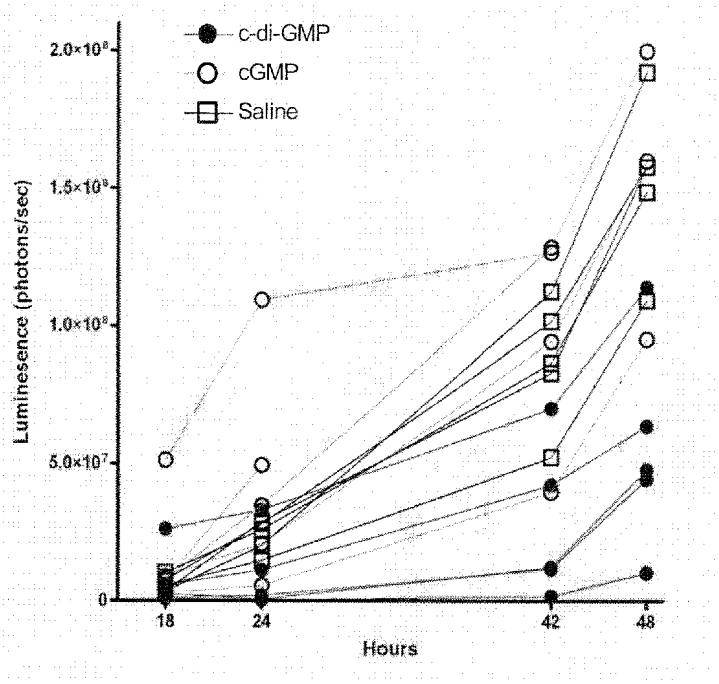

c-di-GMP
cGMP
Saline
Luminescence (photons/sec)
2.0×10⁸
1.5×10⁸
1.0×10⁸
5.0×10⁷
0
18
24
42
48
Hours

Figure 1B

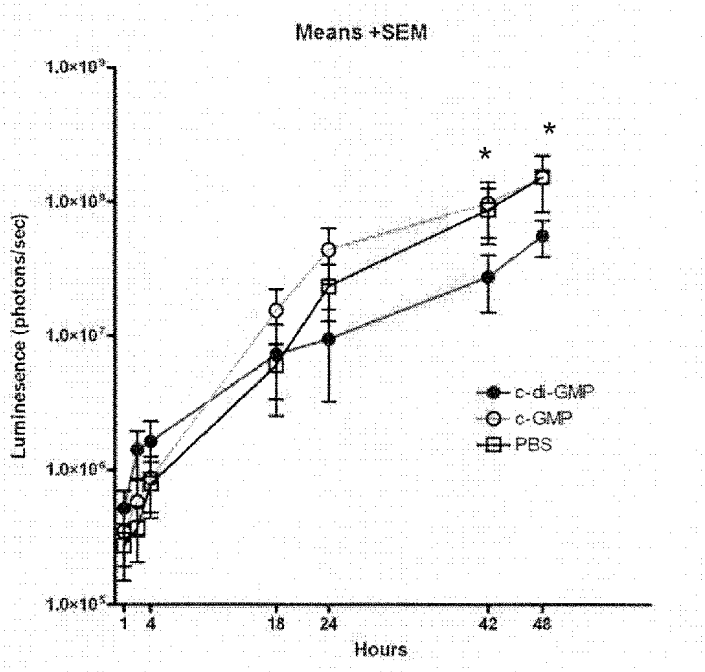

Means +SEM
Luminescence (photons/sec)
1.0×10⁹
1.0×10⁸
1.0×10⁷
1.0×10⁶
1.0×10⁵
1
4
18
24
42
48
Hours
*
*
c-di-GMP
c-GMP
PBS

Figure 1C

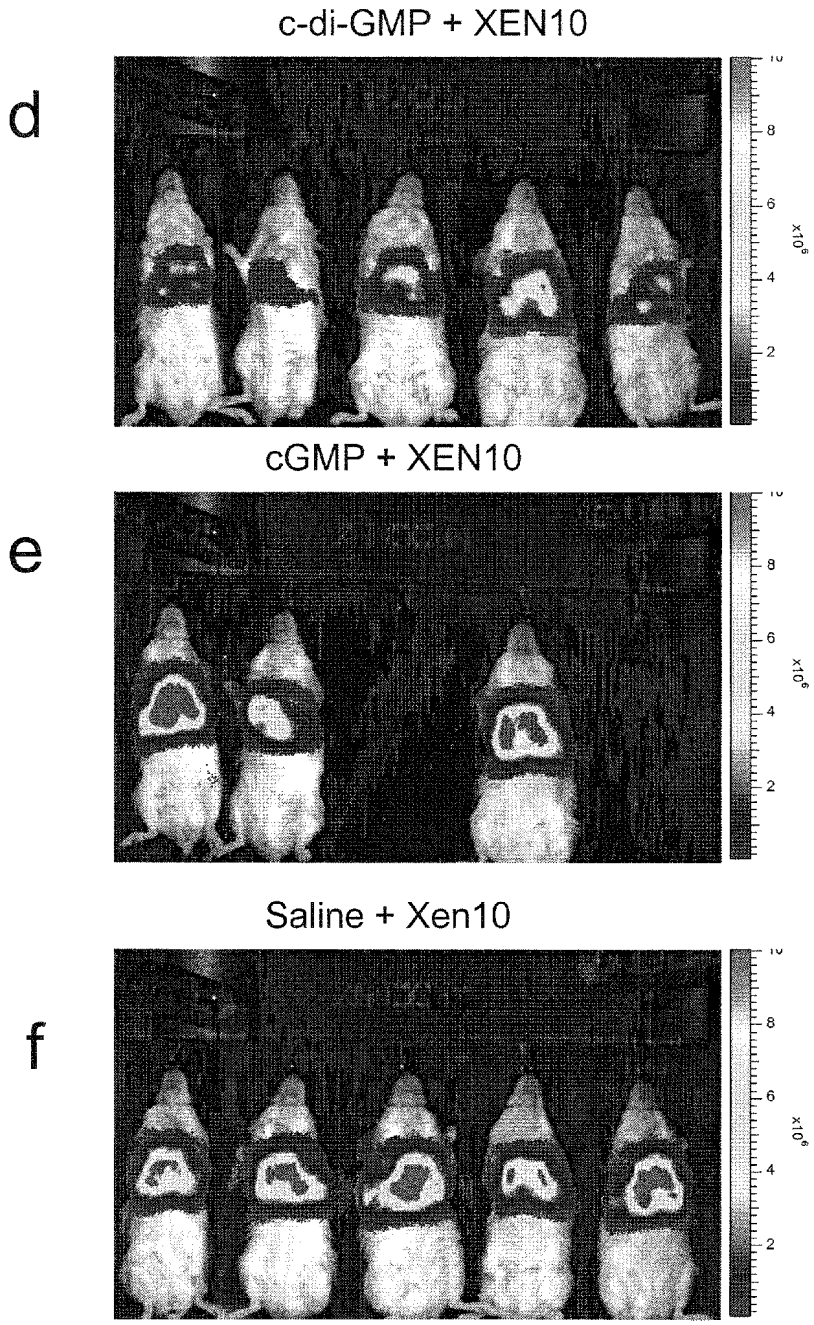
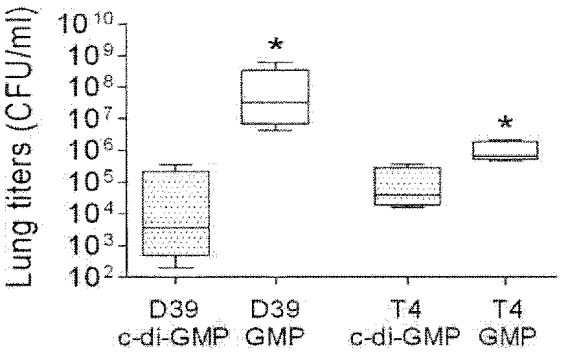
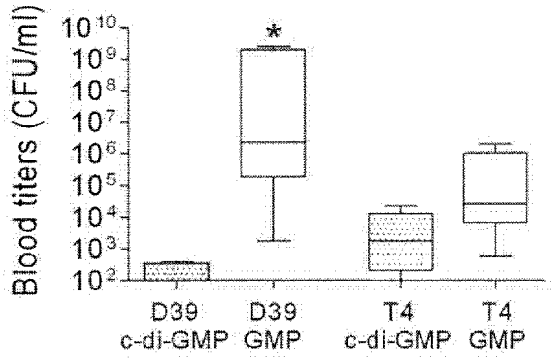


Figure 1 D, E, F

A


Lung titers (CFU/ml)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
*
*
D39 c-di-GMP
D39 GMP
T4 c-di-GMP
T4 GMP

B


Blood titers (CFU/ml)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
*
D39 c-di-GMP
D39 GMP
T4 c-di-GMP
T4 GMP

C

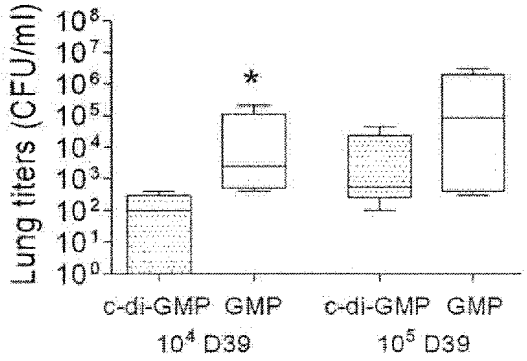

Lung titers (CFU/ml)
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
*
c-di-GMP
GMP
c-di-GMP
GMP
10^{4} D39
10^{5} D39

Figure 2

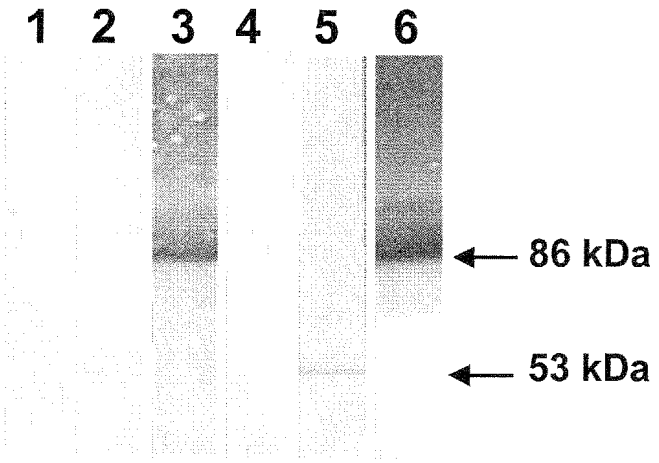


Figure 3

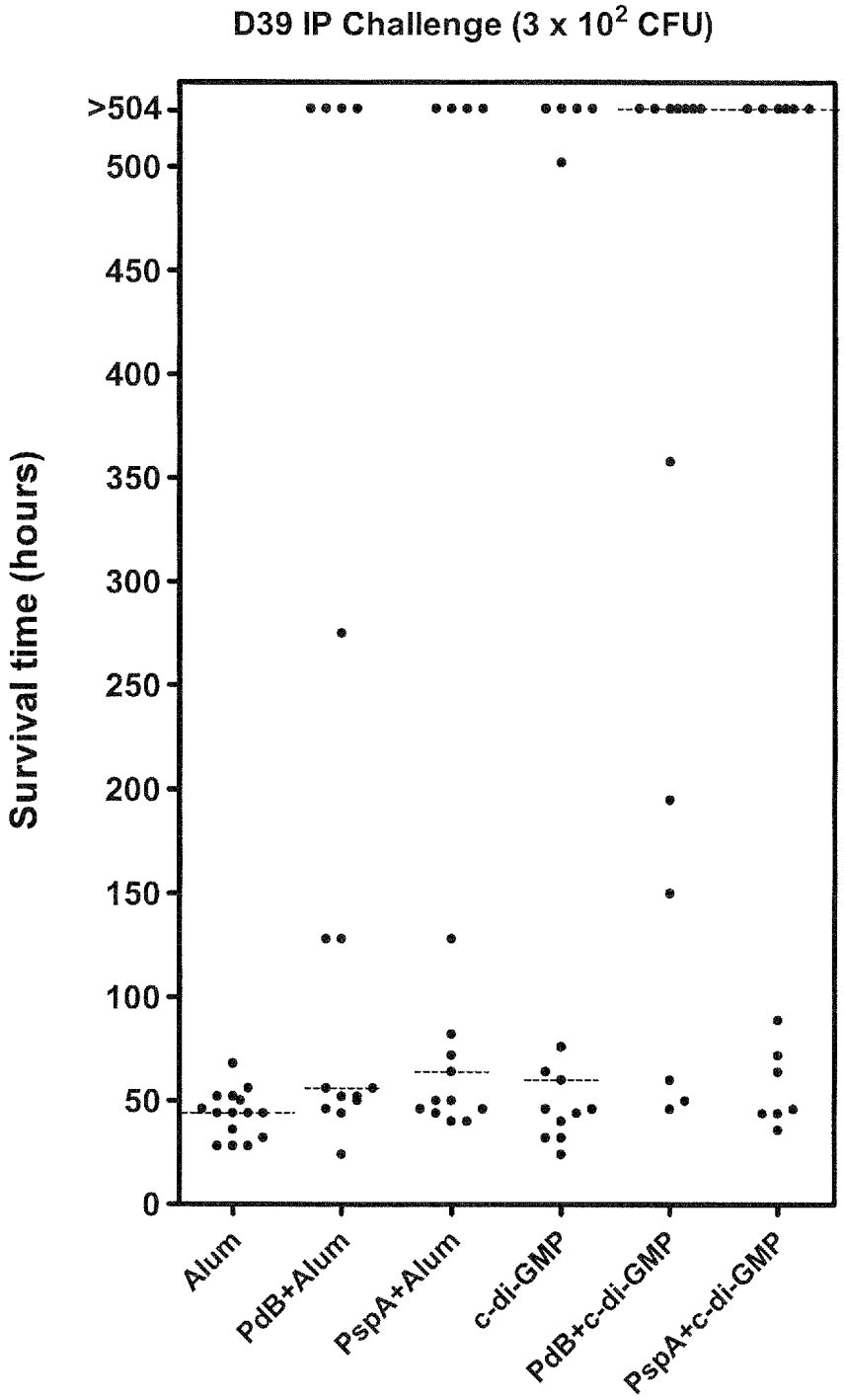

D39 IP Challenge (3 x 10^2 CFU)
Survival time (hours)
>504
500
450
400
350
300
250
200
150
100
50
0
Alum
PdB+Alum
PspA+Alum
c-di-GMP
PdB+c-di-GMP
PspA+c-di-GMP

Figure 4

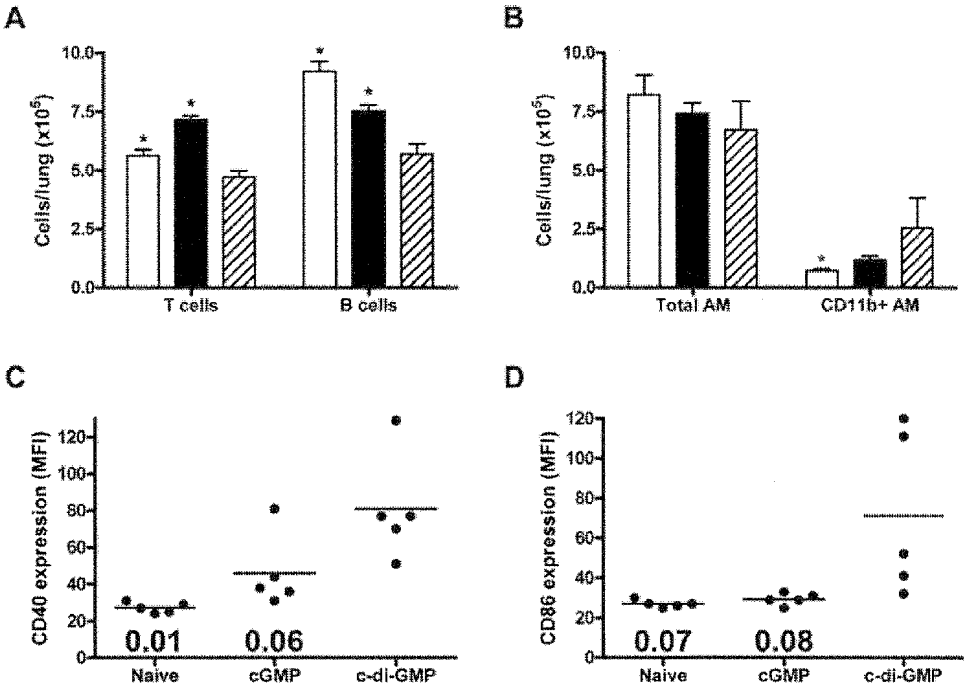

A
Cells/lung (x10^5)
10.0
7.5
5.0
2.5
0.0
T cells
B cells
B
Cells/lung (x10^5)
Total AM
CD11b+ AM
C
CD40 expression (MFI)
120
100
80
60
40
20
0
0.01
0.06
Naive
cGMP
c-di-GMP
D
CD86 expression (MFI)
0.07
0.08
Naive
cGMP
c-di-GMP

Figure 5

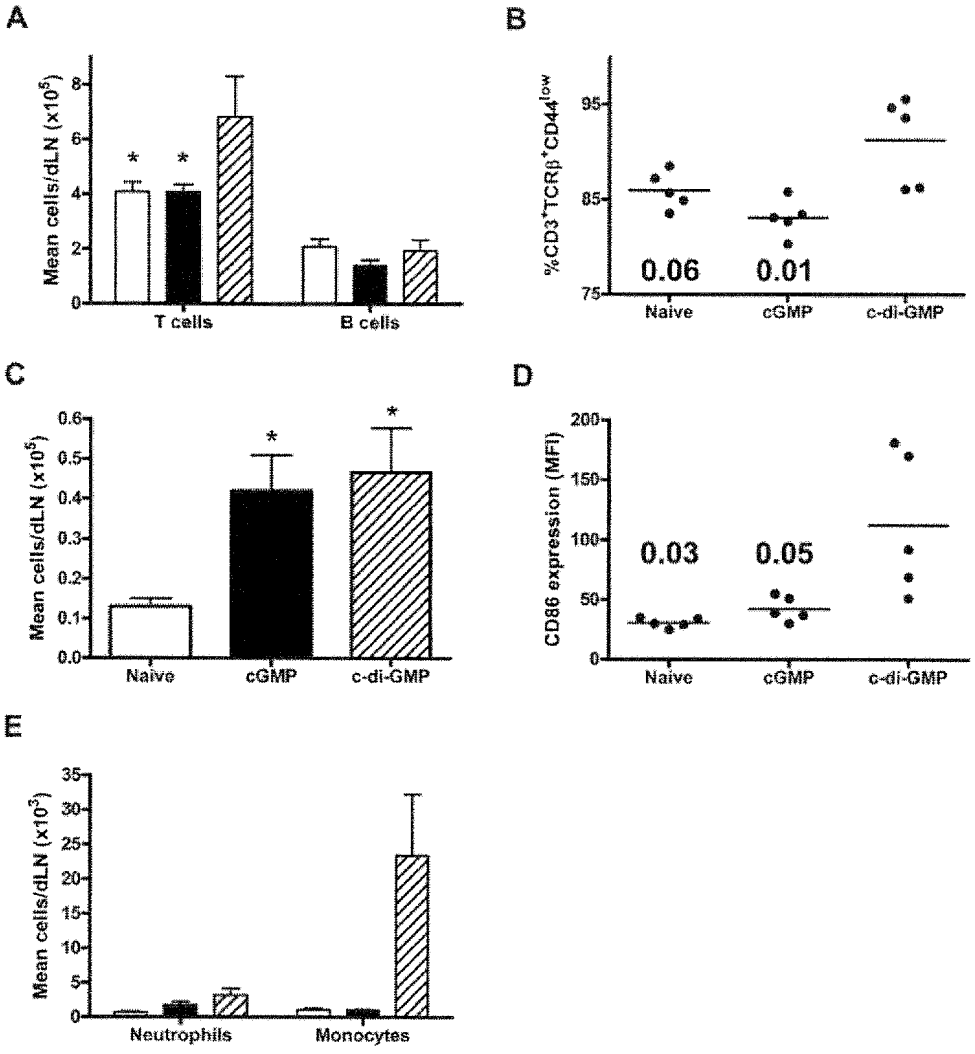

A
Mean cells/dLN (x10^5)
T cells
B cells
B
%CD3+TCRβ+CD44low
0.06
0.01
Naive
cGMP
c-di-GMP
C
Mean cells/dLN (x10^5)
Naive
cGMP
c-di-GMP
D
CD86 expression (MFI)
0.03
0.05
Naive
cGMP
c-di-GMP
E
Mean cells/dLN (x10^3)
Neutrophils
Monocytes

Figure 6